EuroFlow Standard Operating Procedure

(SOP) for

sample preparation and staining

Version 1.2.1

11 November 2015

Updated in Version 1.2.1

- Several text adaptations have been introduced to improve clarity and avoid misinterpretation
- Adjustment of pH and BSA concentration of washing buffer. Effect of pH and BSA concentration was reassessed. Washing buffer pH set at 7.4 result in a reduction on data variability. Lower BSA concentration does not impact the staining pattern while it helps to reduce costs.
- Acquisition of cells in PBS (without NaN_3), to prevent NaN_3 accumulation in the wasting container

For complementary information or questions, please go to
www.euroflow.org or contact Juan Flores-Montero at jflores@usal.es.

EuroFlow SOP for Sample preparation and staining

Content

1.	General remarks	3
2.	Common initial procedure when the EuroFlow antibody panel includes surface membrane (Sm) immunoglobulins (Ig) staining	4
3.	Staining of backbone markers	5
4.	Staining of surface markers only	6
5.	Combined staining of intracellular and surface membrane markers	7
6.	Nuclear (Nu) TdT staining (tube 4 AML/MDS EuroFlow panel)	8
7.	Overview of protocol sections for the various EuroFlow antibody panels and corresponding tubes	9

1. General remarks

- Please comply with all recommendations, even small deviations will impact the results.
- Washing buffer consists of a PBS + 0.09% of NaN_3 + 0.2% BSA filtered solution
- Please note that pH of the washing buffer is recommended to be set and maintained at 7.4
- Please use >98% pure BSA

2. Common initial procedure when the EuroFlow antibody panel includes surface membrane (Sm) immunoglobulin (Ig) staining

If the EuroFlow antibody panel to be applied includes SmIg staining, follow these initial steps; otherwise go directly to the backbone, surface, or intracellular staining protocols (sections 3, 4, 5, respectively):

2.1. Pipette 300 µL of sample into a 10 mL Falcon tube (*see Note 1*).

Note: For small samples (i.e. CSF, vitreous aspirates) spin down the total volume (5 min at 540 g), discard the supernatant (see point 2.5) and resuspend in 300 µL of washing buffer.

2.2. Add 6 mL of washing buffer
2.3. Mix well
2.4. Fill the tube up to 10 mL (by adding 4 mL of washing buffer)
2.5. Mix well
2.6. Centrifuge for 5 min at 540 g
2.7. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet
2.8. Mix well
2.9. Repeat point 2.2 to 2.8
2.10 Repeat point 2.2 to 2.4 (in total 3 washing steps)
2.10. Mix well
2.11. Centrifuge for 5 min at 540 g
2.12. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet
2.13. Mix well
2.14. Resuspend the cell pellet in 300 µL of washing buffer
2.15. Continue with conventional EuroFlow SOPs for staining of cell surface or cell surface plus intracellular markers as described below in Sections 3, 4 and 5, respectively

3. Staining of backbone markers

3.1. Calculate the total volume of surface membrane backbone antibodies based on the number of tubes in the panel (*see Note 2*)

Note: Intracellular backbone markers should not be added at this time

3.2. Pipette these antibodies in one tube (backbone tube)
3.3. Calculate the total volume of sample to be stained, also based on the number of tubes in the panel and a volume of 50 µL per tube
3.4. Pipette this sample volume into the backbone tube
3.5. Mix well
3.6. Pipette equal amounts of the sample-backbone mix into the various tubes included in the applied EuroFlow panel (*see Note 3*).

Note: Both the volume pipetted into each tube and the overall number of tubes depend on the specific EuroFlow panel that is applied

3.7. Continue with the steps described below in Section 4 or 5

4. Staining of surface markers only

Note: PCD tube 2 is processed identically to PCD tube 1 as described in section 5 if CD138-PacO is used

4.1. Add the appropriate volume of antibodies directed against cell surface markers (except for the backbone markers), as recommended for each specific EuroFlow panel
4.2. If necessary, use washing buffer to reach a final volume of 100 µL per tube (see information on the EuroFlow panels)
4.3. Mix well
4.4. Incubate for 30 min at room temperature (RT) protected from light
4.5. Add 2 mL of 1x FACS Lysing Solution (10x FACS Lysing Solution diluted 1/10 vol/vol in distilled water (dH_20) following manufacturers recommendations)
4.6. Mix well
4.7. Incubate for 10 min at RT protected from light
4.8. Centrifuge for 5 min at 540 g
4.9. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 50 µL residual volume in each tube
4.10. Mix well
4.11. Add 2 mL of washing buffer to the cell pellet
4.12. Mix well
4.13. Centrifuge for 5 min at 540 g
4.14. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 50 µL residual volume in each tube
4.15. Mix well
4.16. Resuspend the cell pellet in 200 µL of PBS
4.17. Acquire the cells preferably immediately after staining or store at 4ºC maximally for 1 hour until measured in the flow cytometer

5. Combined staining of Intracellular and Surface membrane markers

Note: Tube 4 of the AML/MDS panel should be stained as described in Section 6

5.1. Add the appropriate volumes of antibodies for cell surface markers, as recommended for each specific EuroFlow panel
5.2. If necessary, use washing buffer to reach a volume of 100 µL per tube (see information on the EuroFlow panels)
5.3. Mix well
5.4. Incubate for 30 min at RT protected from light
5.5. Add 2 mL of washing buffer to the cell pellet
5.6. Mix well
5.7. Centrifuge for 5 min at 540 g
5.8. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 50 µL residual volume in each tube
5.9. Resuspend the cell pellet by mixing gently
5.10. Add 100 µL of Reagent A (fixative; Fix&Perm™, An der Grub, Vienna, Austria)
5.11. Incubate for 15 min at RT protected from light
5.12. Add 2 mL of washing buffer to the cell pellet
5.13. Mix well
5.14. Centrifuge for 5 min at 540 g
5.15. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 50 µL residual volume in each tube
5.16. Resuspend the cell pellet by mixing gently
5.17. Add 100 µL of Reagent B (permeabilizing solution; Fix&Perm™)
5.18. Mix well
5.19. Add the appropriate volume of the intracellular antibodies (see EuroFlow panels)
5.20. Mix well
5.21. Incubate for 15 min at RT protected from light
5.22. Add 2 mL of washing buffer to the cell pellet
5.23. Mix well
5.24. Centrifuge for 5 min at 540 g
5.25. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 50 µL residual volume in each tube
5.26. Mix well
5.27. Resuspend the cell pellet in 200 µL of PBS
5.28. Acquire the cells preferably immediately after staining or store at 4ºC maximally for 1 hour until measured in the flow cytometer

6. Nuclear (Nu)TdT staining (Tube 4, AML/MDS EuroFlow panel)

6.1. Continued from Section 4 step 15
6.2. Add the appropriate amount of the TdT antibody to the cell pellet
6.3. Mix well
6.4. Incubate for 15 min at RT protected from light
6.5. Add 2 mL of washing buffer to the cell pellet
6.6. Mix well
6.7. Centrifuge for 5 min at 540 g
6.8. Resuspend the cell pellet in 200 µL PBS
6.9. Mix well
6.10. Acquire the cells preferably immediately after staining or store at 4ºC maximally for 1 hour until measured in the flow cytometer

Overview of protocol Sections for the various EuroFlow antibody panels and corresponding tubes.

Antibody Panel	Tube(s)	Protocol Procedure				
		2	3	4	5	6
ALOT	1				X	
BCP-ALL	1,4	X	X	X		
	2,3	X	X		X	
T-ALL	1-4		X		X	
AML/MDS	1-3, 5-7		X	X		
	4		X	X		X
LST	1	X		X		
SST	1	X		X		
PCD	1-2		X		X	
B-CLPD	1-4	X	X	X		
T-CLPD	1,2,4,6		X	X		
	3,5		X		X	
NK-CLPD	1,2		X	X		
	3		X		X	

ALOT, Acute leukemia orientation tube; BCP-ALL, B-cell precursor acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia; AML/MDS, Acute myeloid leukemia and myelodysplastic syndrome; LST, Lymphoid screening; SST, small sample tube; PCD, plasma cell disorders; CLPD, chronic lymphoproliferative disorders.